Isolation of Strigol, a Germination Stimulant for *Striga asiatica*, from Host Plants

Bupe A. Siame,† Yohan Weerasuriya,‡ Karl Wood,§ Gebisa Ejeta,‡ and Larry G. Butler*,†

Departments of Biochemistry, Agronomy, and Chemistry, Purdue University, West Lafayette, Indiana 47907

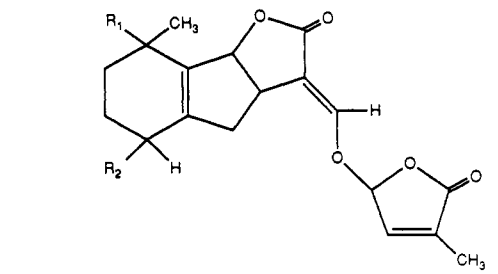
The germination of *Striga asiatica*, a root parasite of many cereal and leguminous crops, is stimulated by several host and nonhost plant derived stimulants. HPLC revealed the presence of three active compounds in root exudates from *Striga* host plants, maize and sorghum, and also from proso millet. A fourth active compound was present in sorghum exudates. Acetate and heptafluorobutyrate derivatives were prepared and analyzed by HPLC and mass spectrometry. Each step involved in the isolation, chromatographic purification, and derivatization was followed by a sensitive *Striga* seed germination bioassay. We report the isolation of strigol as the major *Striga* seed germination stimulant in maize and proso millet root exudates and as a minor component of the total activity in sorghum root exudates. Strigol was previously isolated only from cotton, a nonhost plant.

INTRODUCTION

The genus *Striga* comprises root parasites of many cereal and leguminous crops and causes considerable yield losses of various crops in tropical and subtropical countries (Parker and Reid, 1979). *S. hermonthica* (Del.) Benth and *S. asiatica* (L.) Kuntze are the most virulent species on cereals, whereas *S. gesnerioides* (Willd.) Vatke is the primary parasite on legumes (Parker and Reid, 1979). As part of its adaptation to parasitism, *Striga* species produce large numbers of seeds with prolonged viability and special germination requirements. To germinate, a *Striga* seed must go through an after-ripening process, then pretreatment (conditioning) in a warm moist environment for several days, and finally exposure to an exogenous germination stimulant (Sahai and Shivanna, 1982; Worsham and Egley, 1990).

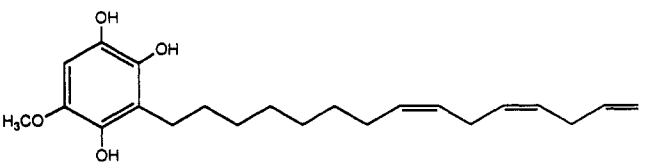
Striga germination stimulants are produced as root exudates by host and nonhost plant species. Strigol (Figure 1a) was isolated from root exudates of cotton (*Gossypium hirsutum* L.), a nonhost plant (Cook et al., 1966, 1972). Strigol was found to be very active, inducing 50% germination of *S. asiatica* at 10^{-11} M. Vail et al. (1990a) tested 15 synthetic terpenoids similar in structure to strigol. Nine of these compounds were active as germination stimulants of *S. asiatica* and *Orobanche ramosa*. In another study four sesquiterpene lactones that share structural features with the lactone rings of strigol were shown to stimulate *Striga* seed germination at concentrations comparable to those of strigol (Fisher et al., 1989). Other compounds reported to stimulate *Striga* seed germination include ethylene (Egley and Dale, 1970), sulfuric acid (Egley, 1972), and sodium hypochlorite (Hsiao et al., 1981).

Several attempts have been made to identify *Striga* germination stimulants produced by host plants. The stimulants partially characterized had some water solubility and were proposed to resemble strigol (Cook et al. 1972; Parker, 1983). However, the first host-derived *Striga* germination stimulant identified was sorgoleone (Chang et al., 1986), which has very low solubility in water. The active form of sorgoleone (Figure 1c) was found to be the unstable dihydroquinone, which was rapidly oxidized to the inactive quinone. An agar gel assay (Hess et al., 1992) and a dilution assay (Weerasuriya et al., 1993) for germination stimulants showed great differences in the amount of stimulants produced by different sorghum cultivars. However, resistant sorghum cultivars whose resistance to *Striga* was based on low stimulant production were found to produce the same amounts of sorgoleone as the most susceptible cultivars. The agar gel assay seems to measure stimulants that are more water-soluble and more stable than sorgoleone. Its results correlate well with field reports of resistance/susceptibility based on different levels of stimulant production as measured by other investigators (Vasudeva Rao, 1987). Therefore, Hess et al. (1992) concluded that *Striga* seed germination is largely controlled by stimulants more water-soluble than sorgoleone.



(a) $R_1 = CH_3$, R_2 = OH, Strigol

(b) $R_1 = R_2$ = H, Sorgolactone



(c) Sorgoleone

Figure 1. Structures of (a) strigol, (b) sorgolactone, and (c) sorgoleone.

Sorgolactone (Figure 1b), a compound closely related to strigol, was recently reported to be the major *Striga* seed germination stimulant from sorghum root exudates (Hauck et al., 1992). We have confirmed earlier reports

† Department of Biochemistry.
‡ Department of Agronomy.
§ Department of Chemistry.

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that *Striga* host plants produce several compounds which stimulate *Striga* seed germination (Egley, 1990). We now report that strigol is the major *Striga* germination stimulant in the root exudate of maize (*Zea mays* L.) and proso millet (*Panicum miliaceum* L.). A small amount of strigol is also produced by sorghum (*Sorghum bicolor* L. Moench). Strigol was not detected in cowpea (*Vigna unguiculata* L. Walp) root exudates.

MATERIALS AND METHODS

Sources of Seeds and Strigol. Seeds of *S. asiatica* were obtained from Dr. R. Eplee (USDA/APHIS, Whiteville Method Development Center, Whiteville, NC). Seeds were stored and handled under quarantine restrictions approved by USDA/APHIS and the Indiana Department of Natural Resources. A commercial maize hybrid (Agrotech 825) and a germplasm sorghum line (IS 4225) were grown at the Purdue University Agronomy Research Center. Proso millet seeds were obtained from a commercial source (marketed as Morning Song Bird Feed by Gutwein Milling Co. Inc., Francesville, IN). Cowpea seeds were obtained from Dr. Murdock, Purdue University. Standard (±)-strigol was obtained from Dr. R. J. Riopel (University of Virginia, Charlottesville, VA).

Surface Sterilization of Seeds. *S. asiatica* seeds were surface-sterilized and conditioned as described previously (Hess et al., 1992). Cowpea, maize, proso millet, and sorghum were surface-sterilized and germinated as reported by Hess et al. (1992) with minor changes in the method. Proso millet seeds were soaked overnight in water containing 5% instead of 10% Captan 50-WP. Cowpea, sorghum, and proso millet seeds were germinated for 24 h and maize seeds for 48 h prior to planting.

Production and Collection of Root Exudates. Stimulant production and collection were done as described by Weerasuriya et al. (1993). Root exudates (50–80 mL from each tube) were collected daily over a period of 7 days and stored in a refrigerator.

Striga Seed Bioassay. The bioassay was conducted in Falcon multiwell plates (Becton Dickinson Co., Lincoln Park, NJ) containing 0.5 mL of the test solution. Conditioned *Striga* seeds (50–100) were placed in each well and incubated at 28 °C. Germinated and ungerminated seeds were counted 24 h later under a dissecting microscope. All tests were performed in duplicates.

Extraction and Cleanup of Stimulants from Root Exudates. Proso millet root exudates collected over 7 days were pooled and extracted with ethyl acetate. One-liter portions were extracted with 3 × 400 mL of ethyl acetate. The ethyl acetate from the extraction of 10 L of root exudates was evaporated, and the residue was taken up in 5 mL of methanol/water (1:1 v/v). Root exudates from the other crops were extracted in a similar way. Strigol (20 mL of a 10^{-4} M aqueous solution) was also extracted with ethyl acetate (3 × 2 mL). Ethyl acetate was evaporated and the residue taken up in 1 mL of methanol/water (1:1 v/v).

A glass column 25 × 500 mm was filled with a slurry of Sephadex LH20 (Pharmacia Fine Chemicals Inc., Piscataway, NJ) in methanol/water (1:4 v/v) to the 250-mm mark. The methanol/water extract of the stimulant from each crop was loaded on the column and eluted with methanol/water (1:4 v/v) at a flow rate of 0.4 mL/min. One hundred fractions were collected in 20-min intervals. The UV absorption of the fractions collected was monitored at 240 nm on a Gilford Response spectrophotometer (Ciba Corning Diagnostics Corp., Medfield, MA). Fractions were diluted 1:10 successively and bioassayed using conditioned *S. asiatica* seeds. Fractions containing active stimulants were combined, solvents were evaporated, and the residue was taken up in 2 mL of methylene chloride.

Further purification of the stimulants was done on Waters μBondapack C_{18} analytical (3.9 × 300 mm) and preparative (19 × 300 mm) HPLC columns connected to a Varian 5000 LC system equipped with a Hewlett-Packard HP1040A photodiode array detector. Samples were 0.05 and 0.50 mL on the analytical and preparative columns, respectively. The elution gradient was 100% water to 100% acetonitrile in 50 min. Flow rates were 1.5 and 4.0 mL/min for the analytical and preparative columns, respectively. UV absorption of the column effluents was monitored at 240 nm. Fractions were collected in test tubes, diluted 1:10 successively, and then bioassayed. Active fractions from the preparative column were combined, solvents were evaporated, and the residue was taken up in methylene chloride (1 mL) for derivatization and further analysis.

Table I. Efficiency of Extracting Stimulants into Ethyl Acetate (EtAc) from Aqueous Root Exudates of Sorghum[a]

	germination, %				
dilution	before EtAc extrn	after 1st EtAc extrn	after 2nd EtAc extrn	after 3rd EtAc extrn	combined EtAc extract
1×	70	66	73	5	60
1:10	84	72	13	0	82
1:100	81	23	0	0	85
1:1000	79	0	0	0	81
1:10000	42	0	0	0	37
1:100000	20	0	0	0	15

[a] Successive 1:10 dilutions of the aqueous layer remaining after each extraction with ethyl acetate were bioassayed.

Derivatization. Preparation of the acetate derivative of strigol was based on the method of Frischmuth et al. (1991). Acetic anhydride (0.10 mL) and 2 mg of *p*-(dimethylamino)-pyridine were mixed with 1 mL of the HPLC-purified stimulants (in methylene chloride) in a capped vial. The reaction mixture was left to stand at room temperature for 2 h and then washed with water (3 × 1 mL). The methylene chloride layer was separated on preparative HPLC. Fractions with activity were combined, solvents were evaporated, and the residue was taken up in methylene chloride (1 mL).

The heptafluorobutyrate derivative was prepared by evaporating the solvents from 1 mL of the HPLC-purified materials (strigol or sample extract) and taking the residue up in 1 mL of 0.05 M triethylamine in methylene chloride. Heptafluorobutyric anhydride (0.10 mL) was added and the mixture heated in a capped vial for 30 min at 50 °C (Knapp, 1979). The mixture was cooled and washed with water (3 × 1 mL). The methylene chloride layer was separated on preparative HPLC. Fractions with activity were combined, solvents were evaporated, and the residue was taken up in 1 mL of methylene chloride for further analysis.

Esterase Enzyme Reaction. HPLC-purified strigol acetate (1 mL) was placed in a boiling tube, methylene chloride was evaporated, and the residue was taken up in 1 mL of phosphate buffer (pH 7.5). To the mixture was added 0.1 mL of rabbit liver esterase (40 units, 4.6 mg/mL). Albumin (4.4 mg/mL) was used as a control. The mixture was incubated in a water bath at 25 °C for 30 min. After 30 min, the tube was placed in a boiling water bath for 2 min to deactivate the enzyme. Samples were extracted into ethyl acetate (3 × 1 mL), and solvents were evaporated off and residue taken up in methylene chloride and analyzed by HPLC.

Mass Spectrometry. Electron impact and positive and negative chemical ionization mass analyses were carried out using a Finnigan 4000 mass spectrometer (Finnigan-MAT, San Jose, CA). A direct insertion probe heated to 300 °C was used for sample introduction. Ionization energy and emission current were 70 eV and 350 μA, respectively.

RESULTS

On the basis of the bioassay results, active compounds from plant root exudates were effectively partitioned into ethyl acetate from the aqueous phase. After three extractions, the combined ethyl acetate extract showed a stimulant activity profile very similar to that observed in the root exudate before the extraction. Table I shows the stimulant activity of sorghum root exudates after each ethyl acetate extraction. Similar results (not shown) were obtained for the other crops and standard strigol.

Normal-phase column chromatography on silica gel (E. Merck, Darmstadt, Germany) resulted in considerable loss in the activity of the stimulants. Sephadex G10, G20, and G50 (Pharmacia) did not provide any separation of

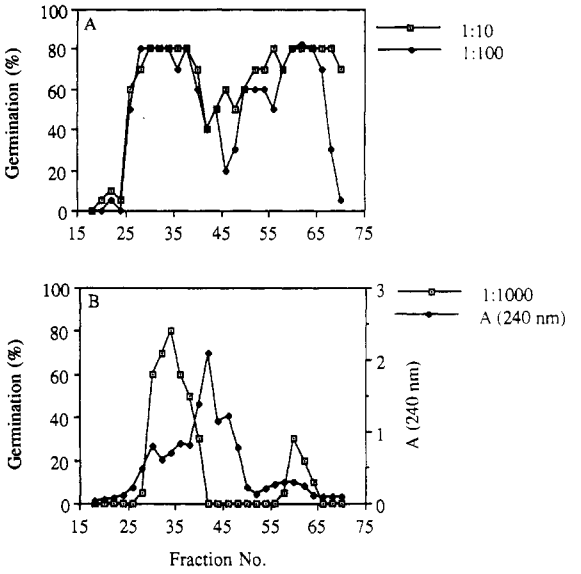

Figure 2. Stimulant activity of proso millet exudates separated on Sephadex LH20. The fractions were eluted with methanol/water (1:4 v/v), and fractions (8 mL/20 min) were collected. Fractions (10 μL) were diluted to 0.5 mL and bioassayed. Successive 1:10 dilutions of the 0.5-mL samples were made and bioassayed. (A) Germination results of the 1:10 and 1:100 dilutions; (B) germination results of the 1:1000 dilutions and the UV absorption of the fractions monitored at 240 nm.

stimulants. Sephadex LH20 proved to be a more useful solid phase. The stimulants were effectively recovered from the Sephadex LH20 (on the basis of the activity before and after Sephadex LH20 separation). The stimulants were also partially separated on the Sephadex LH20 column. Successive 1:10 dilutions were done to determine the most active fractions (Figure 2). Several other UV-aborbing compounds were also present in the exudates (Figure 2B). Two separate fractions with activity were collected from the Sephadex LH20 column for each cereal. Only one active fraction was collected from cowpea root exudates. The compounds in the first active fraction off the column were more active in exudates from maize and proso millet, while the compounds in the second fraction were more active in sorghum exudates. Strigol eluted from the column in the same volume as the first active fraction from the three crops.

The first active fraction off the Sephadex LH20 column was separated further on an analytical HPLC column. Three active fractions were observed in the cereal exudates, with retention times (Rt) of 21, 27, and 31 min, henceforth referred to as fractions A, B, and C, respectively (Figure 3). Fraction B was the most active in exudates from maize and proso millet. This fraction was active at 100–1000-fold greater dilution than the other fractions. When the second active fraction off the Sephadex LH20 column was separated on HPLC under the same conditions as the first fraction, two active fractions were observed in the extracts from maize and proso millet with Rt of 27 and 31 min. These were later shown to be the same as fractions B and C, respectively. In addition to these two active fractions, a third active fraction, D, with Rt of 34 min was often observed in exudates from sorghum (Figure 4). Fraction C was the most active in exudates from sorghum. Only fraction A was detected in cowpea exudates.

Samples from Sephadex LH20 were purified by preparative HPLC and fractions with the respective active fractions collected. Sample fraction B off the HPLC (Rt of 27 min) had chromatographic properties identical to those of strigol under several different conditions. Further

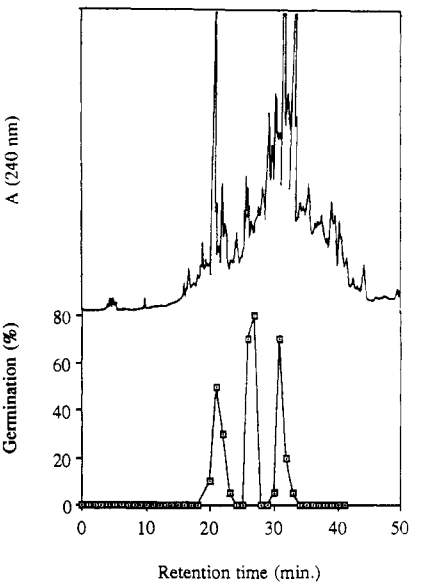

Figure 3. HPLC of the proso millet exudates separated on Sephadex LH20. The first active fraction off the column was further separated on a Waters μBondapack C_{18} column (3.9 × 300 mm). A solvent gradient from 100% water to 100% acetonitrile in 50 min was used. The UV absorption was monitored at 240 nm. Fractions were collected every minute at a flow rate of 1.5 mL/min. Successive 1:10 dilutions were made and bioassayed. Three active fractions were observed with Rt 21, 27, and 31 min in all crops. The figure shows germination results for the 1:1000 dilutions.

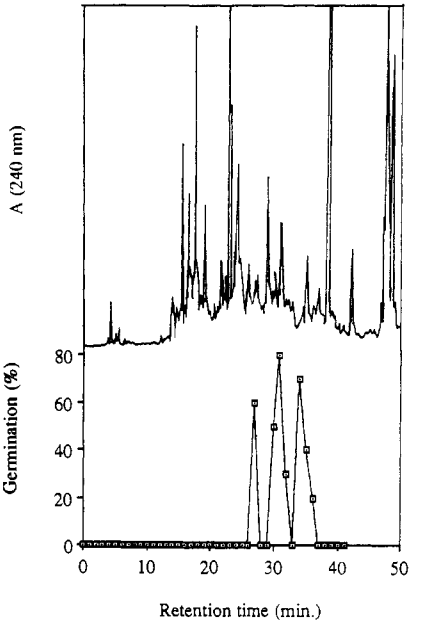

Figure 4. Second fraction with activity off the Sephadex LH20 column was analyzed by HPLC as in Figure 3. Three fractions with activity (Rt 27, 31, and 34 min) in sorghum exudates were observed. Germination results for the 1:1000 dilutions are shown.

experiments were done to determine whether the active compound was actually strigol. Strigol has a hydroxyl group which can be derivatized, whereas sorgolactone does not (Figure 1). Acetylation of strigol and analysis by HPLC revealed a shift in the elution time of both the absorbance peak and the activity from Rt of 27 min to Rt of 31 min (Figures 5 and 6). Acetylation of sample fraction B, which had chromatographic properties similar to those of strigol, similarly showed an increase in activity at Rt of 31 min and a decrease in the activity at Rt of 27 min (Figures 7 and 8). However, it was not possible to get extracts of fraction B stimulant completely free of fraction C activity.

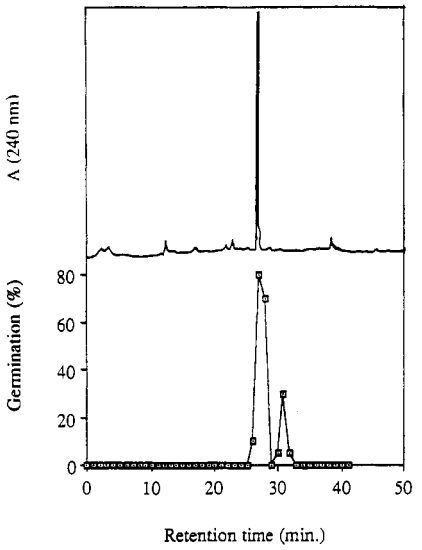

Figure 5. HPLC of standard strigol. The conditions are as given in Figure 3. The strigol absorbance peak and activity had Rt of 27 min.

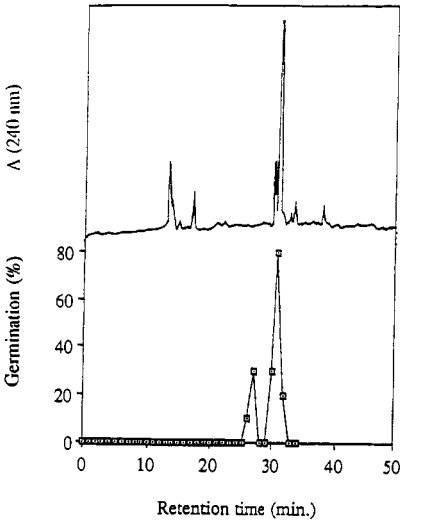

Figure 6. HPLC of standard strigol after derivatization with acetic anhydride. The absorbance peak and the activity shifted from Rt 27 to 31 min upon acetylation.

When the acetylated strigol (Rt 31 min) was reacted with the rabbit liver esterase to hydrolyze the acetate ester, the absorbance peak and the activity reappeared at Rt 27 min (data not shown). However, considerable reduction in the peak size and activity were observed. This was most probably due to the enzyme hydrolyzing the lactone rings in addition to the acetate ester. Treatment of the acetylated sample fraction B with the rabbit liver esterase decreased activity at Rt 31 min and increased activity at Rt 27 min. Neither fraction A nor fraction C responded to the acetylation–deacetylation reactions. Apparently both of them lack a free hydroxyl group. It was concluded that the sample fraction C was not strigol acetate even though it had the same Rt as acetylated standard strigol. However, treatments with the esterase enzyme resulted in decreased activity of fraction C (Rt 31 min), suggesting that it may have lactone rings or other esters.

Derivatization of strigol with heptafluorobutyric anhydride shifted both the absorbance peak and the activity to a new position with Rt of 38 min (Figure 9). Reacting sample fraction B, putative strigol, with heptafluorobutyric anhydride decreased the stimulant activity at Rt 27 min and new stimulant activity appeared at Rt 38 min (Figure

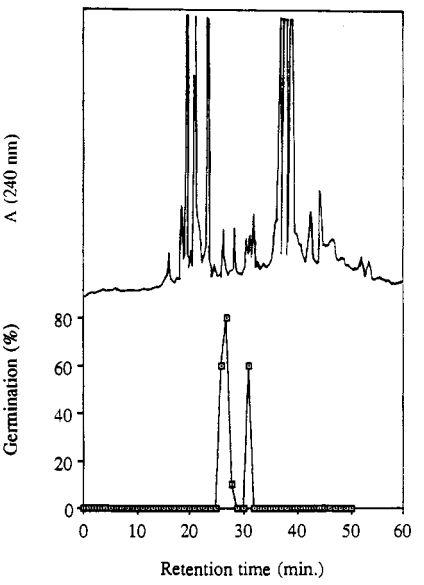

Figure 7. Preparative HPLC purified proso millet exudates corresponding to fraction B (Rt 27 min) were run on an analytical column as in Figure 3. Eluates were monitored at 240 nm. Fractions were collected and bioassayed. Activity was observed at Rt 27 and 31 min.

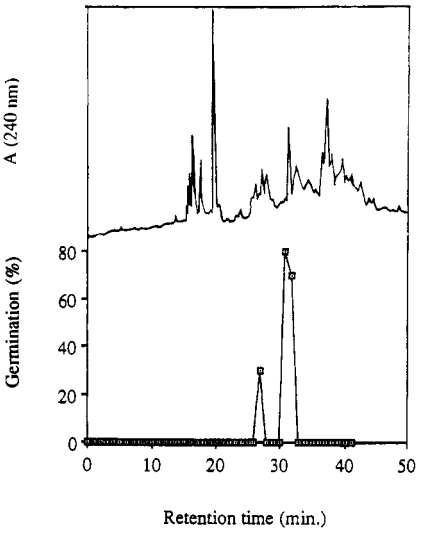

Figure 8. Preparative HPLC purified proso millet exudates corresponding to fraction B (Rt 27 min) were acetylated and analyzed by HPLC. Eluates were monitored at 240 nm. Fractions were collected and bioassayed. There was an increase is stimulant activity at Rt 31 min with a corresponding reduction in activity at Rt 27 min.

10). Thus, fraction B has chromatographic properties identical to those of strigol, and after esterification with both anhydrides, the esters chromatograph identically to those of strigol.

Electron impact (EI) mass spectra of standard strigol were consistent with that reported already (Cook et al., 1966). However, exudate strigol could not be detected by this method. Negative ion chemical ionization (NICI) of the heptafluorobutyrate derivatives (molecular weight 542) was more sensitive and subject to less interference from impurities in the exudate. Figure 11 shows the NICI mass spectra of the derivatized standard strigol (Figure 11A) and derivatized exudate strigol (Figure 11B), respectively. The ion at m/z 445 corresponds to the loss of $C_5H_5O_2$ from the molecular ion of the derivative. This fragmentation, corresponding to the loss of the terminal lactone ring, is consistent with the primary fragment ion observed in EI. The signal obtained for standard strigol derivative cor-

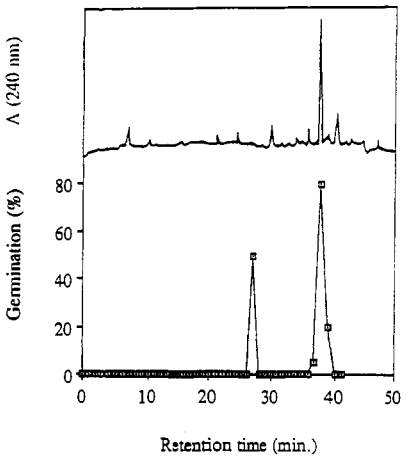

Figure 9. HPLC of the heptafluorobutyrate derivative of standard strigol. The absorbance peak and the activity shifted from Rt 27 to 38 min (a position with no prior activity).

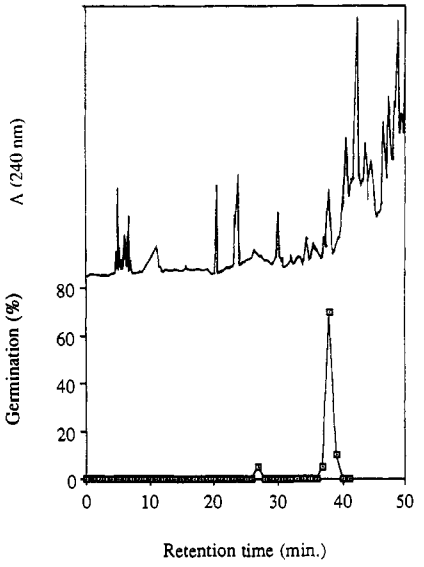

Figure 10. Preparative HPLC purified proso millet exudates with activity observed at Rt 27 min (fraction B) were derivatized with heptafluorobutyric anhydride and analyzed by HPLC. Fractions were collected and bioassayed. A decrease in activity was observed at 27 min, while activity appeared at a new position with Rt 38 min.

responds to approximately 10 ng of strigol. The exudate strigol derivative signal is approximately 500 times less intense, suggesting the mass spectrum shown in Figure 11B resulted from 20 pg of strigol. Other prominent peaks in the NICI spectra, m/z 150, 194, and 213, can be related to the heptafluorobutyrate portion of the derivative.

DISCUSSION

On the basis of chromatographic properties of strigol and its derivatives and the mass spectra of strigol derivatives, we report the isolation of strigol from root exudates of maize, proso millet, and sorghum. Although strigol has been suspected to be present in root exudates of host plants (Cook et al., 1972; Parker, 1983; Egley, 1990), this is the first conclusive report on its occurrence in exudates from natural host plants for *S. asiatica*. Strigol was found to be the major stimulant isolated from maize and proso millet under the conditions used in this study. The major stimulant from sorghum had chromatographic properties similar to those of strigol acetate but did not undergo deacetylation when reacted with rabbit liver esterase. A compound, sorgolactone, differing from strigol in that it lacked the hydroxyl and one methyl group, was recently isolated from sorghum (Hauck et al., 1992). It is likely that our major stimulant from sorghum is sorgolactone. The other stimulant peaks observed may also be structurally related to strigol.

A low germination stimulant activity against *S. asiatica* was observed in cowpea root exudates. However, the chromatographic properties of the cowpea stimulants differed from those of strigol (data not shown). The most active stimulant peak in cowpea exudates had the same retention time on HPLC as fraction A from cereal exudates. A compound named alectrol was recently identified as the major stimulant for *Alectra vogelii* (Benth) and *Striga gesnerioides* from root exudates of cowpea (Muller et al., 1992). The proposed structure for alectrol, a strigol analog, contains a tertiary hydroxyl group which may be shielded from reaction with our esterifying reagents.

The level of strigol in all of the extracts was very low, such that even concentrating the exudate 10000-fold did not yield a useful UV spectrum. Despite this, the extraction and derivatization could be followed by the very sensitive bioassay for stimulation of germination of *S. asiatica* seeds. The low levels at which these stimulants are active reaffirm the suggestion that strigol and strigol-like stimulants may be a new class of plant hormones (Cook et al., 1972; Hsiao et al., 1981). Strigol has also been shown to stimulate the germination of several other parasitic

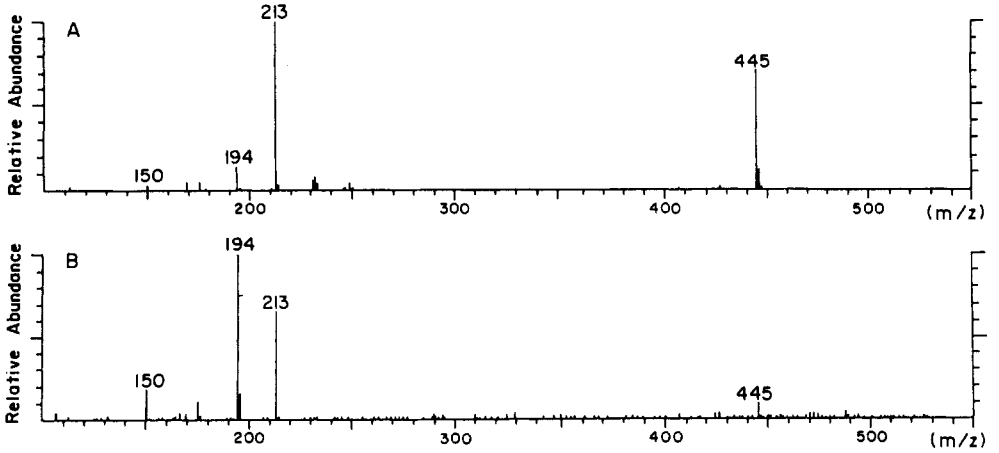

Figure 11. Preparative HPLC purified heptafluorobutyrate derivatives of standard strigol and exudate fraction B (putative strigol) were analyzed by negative ion chemical ionization (NICI) mass spectrometry. (A) NICI mass spectrum of standard strigol; (B) NICI mass spectrum of exudate strigol.

flowering plants at concentrations as low as 10^{-11} M (Hauck and Schildknecht, 1990).

Much effort has gone into identification of *Striga* seed germination stimulants with a view that synthetic compounds based on natural stimulants could be used to control *Striga* by stimulating suicidal germination. Our results show that strigol is the major stimulant in maize and proso millet root exudates and is also produced by sorghum, while sorgolactone (a strigol analog) has been shown to be the major stimulant in sorghum root exudates (Hauck et al., 1992). We are confident that the minor stimulants in our exudates are also structurally related to strigol. The identification of strigol in root exudates from host plants has taken such a long time mainly because most studies have been done with sorghum root exudates in which strigol is only a minor stimulant. The other reason is that it is present in very low concentration in host plant root exudates.

Attempts have been made to use strigol and synthetic strigol analogs to germinate *Striga* under field conditions, but they have proved to be unstable (Vail et al., 1990b). The control of *Striga* based on suicidal germination brought about by natural and/or synthetic analogs of natural germination stimulants does not appear to be feasible at this time.

The mechanism by which strigol stimulates *Striga* seed germination is just now beginning to emerge. Work done in our laboratory (Babiker et al., 1993) and elsewhere (Logan and Stewart, 1991; Jackson and Parker, 1991) showed that strigol, strigol analogs, and sorghum root exudates stimulate ethylene biosynthesis in *Striga* seeds. Further studies are needed to investigate the role of the other germination stimulants on the ethylene biosynthetic pathway in *Striga* seeds.

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